Cytocompatibility and 3D biodistribution with oxidized nanographene assessed by digital holographic microscopy

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ABSTRACT

Nano graphene-based materials offer interesting physicochemical and biological properties for biotechnological applications due to their small size, large surface area and ability to interact with cells/tissues. Among carbon-based nanomaterials, graphene oxide is one of the most used in biological field. There is an increasing interest in shedding light on the interaction mechanisms of nanographene oxide (nGO) with cells. In fact, the effects on human health of GO, and its toxicological profile, are still largely unknown. Here we show that, by minimizing the oxidation degree of GO, its toxicity is significantly reduced in NIH 3T3 cells. Moreover, we show that mild oxidation of graphene nanoplatelets produces nGO particles, which are massively internalized into the cell cytoplasm. MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed to analyze cell viability. Transmission electron microscopy (TEM) analysis was performed to evaluate nGO internalization mechanism into the cytoplasm under different oxidation degree and concentrations. For the first time, we evaluated quantitatively, the cell volume variation after nGO internalization in live fibroblasts through a label-free digital holography (DH) imaging technique and in quasi-real-time modality, thus avoiding the time-consuming and detrimental procedures usually employed by electron-based microscopy. In conclusion, here we have demonstrated that DH can be a viable tool to visualize and display 3D distributions of nano graphene oxide (nGO) uptake by fibroblast cells. DH opens the route for high-throughput investigation at single cell level for understanding how in different conditions nanoparticles aggregates distribute inside the cells.

Keywords: cells, nano graphene oxide, digital holography microscopy, drug delivery, nanocarrier, cellular uptake, cytotoxicity.

1. INTRODUCTION

In the last decade, the two-dimensional material graphene has captured much attention due to its superb electronic properties [1,2] and promising applications in biomedicine field, including approaches to fight or detect coronavirus disease 19 (COVID-19) cases caused by a new coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [3,4]. In the growing area of nanomedicine, graphene-based materials (GBM), and in particular GO, are some of the most recent explored nanomaterials for applications in biotechnology [5]. In particular, the ongoing progress in nanotechnology is rapidly opening the doors for a wide number of advanced biomedical applications. However, it was not until 2008 that graphene-based materials were first introduced into the field of biomedical sciences, when Dai et al. used nanographene oxide (GO) as an efficient nanocarrier for drug delivery [6]. Since then, interest in its biomedical applications has gained momentum, from drug and/or gene delivery, to bioimaging [7], to biosensors [8] and biomedical devices for tissue engineering [9]. Given their ability to interact with biomolecules, such as DNA, and protect them from enzymatic degradation, as well as their capacity to act as delivery vehicles in living cells and *in-vivo* systems, graphene and its derivatives have firmly established themselves as next-generation candidates for biotechnological advancements. Graphene derives its properties from its chemical structure, which comprises a flat monolayer of carbon atoms packed into a 2D honeycomb lattice; this lattice is a basic building block for all other graphitic materials. Among graphene family nanomaterials (GFNs), graphene oxide (GO), which is produced by oxidation of graphite to graphite oxide, is considered the most versatile graphene-based derivative, and is increasingly attracting attention in biomedical field. GO, an oxidation form of graphite, usually has single, bi- or multilayers of graphene sheets with epoxide, hydroxyl, and carboxylic acid groups [10]. GO is commonly synthesized using the Hummers method or derivatives therefore [11,12,13], which involves oxidation of graphite to various levels by using potassium permanganate and sulfuric acid. GO contains a range of oxygen functional groups, mainly hydroxyl and epoxy groups on the basal plane, with smaller amounts of

carboxyl, carbonyl, phenol, and lactone at the sheet edges [14,15,16,17]. The hydroxyl and epoxy groups form hydrogen bonds and weak interactions with other groups, whereas the carboxylic acid group offers negative surface charge and stability in polar solutions [18]. Thus, GO disperses well in water and other polar solvents.

However, the biological behaviour of graphene and its derivatives especially their interactions with different cellular systems and their effects on the human health are still largely unknown. [19,20,21]

Among GFNs, researchers selected graphene sheets (GS) and GO to study their interaction with a biological environment. Liao et al. [22] have studied the blood compatibility and cytotoxicity of GO and GS of various sizes and oxygen content in suspended human red blood cells (RBCs) and adherent skin fibroblasts using *in-vitro* haemolysis and WST-8 viability assay. They found that at the smallest size, GO showed the greatest hemolytic activity, whereas aggregated GS exhibited the lowest hemolytic activity on RBCs. Coating GO with chitosan nearly eliminated hemolytic activity. Together, these results demonstrate that particle size, particulate state, and oxygen content/surface charge of graphene have a strong impact on biological/toxicological responses to red blood cells. The toxicity of both systems depends on several properties of the material, such as shape or dimensions. Moreover, their results reveal that GS resulted in a higher cytotoxic behaviour with respect to GO since the compacted graphene sheets are more damaging on human skin fibroblast than the less densely packed graphene oxide. Yun-Jung Choi et al. [23] compared the cytotoxic effect of GO and reduced graphene oxide (rGO) using uric acid (UA) on human ovarian cancer cells, discovering that the toxicity of UA-rGO is significantly higher than GO. Indeed, UA-rGO significantly induces cell death by an increase in lactate dehydrogenase release, generation of reactive oxygen species (ROS), activation of caspase 3 and DNA fragmentation. The toxicological profile of graphene-based nanomaterials is a very important issue, because it holds an increasing interest for a wide variety of biological applications, but it is not yet well understood. Therefore, when GFNs are investigated for *in-vivo* purposes in animals, human body and for food packaging and pharmaceutical industries, their biocompatibility must be considered [24].

Recently, most studies indicated that different parameters such as concentration, shape, chemical functionalization, hydrophobicity, surface charge, type of dispersants, lateral dimension, exposure time, oxidation degrees and cell type can influence the cytotoxicity of graphene and GO[25,26]. Yue at al. [27] have reported a study on the role of lateral dimension comparing micro- and nano-sized GO in macrophages. It resulted in a better biocompatibility of nano-sized GO, with respect to micro-sized GO, which induced a much stronger inflammation response.

Human fibroblasts responded to water soluble graphene oxide – prepared by Hummers method and its modified procedures – in different way depending on dose. Results showed that GO with dose less than 20 µg/ml did not exhibit toxicity to fibroblasts cells, on the other hand, dose of more than 50 µg/ml exhibited cytotoxicity such as decreasing cell adhesion, cell apoptosis, entering cytoplasm, lysosomes and mitochondria [28]. Also, the chemistry of surface is an important factor that plays a critical role in the biocompatibility, since it has been shown that functionalization of graphene can lead to a reduction of reactive oxygen species (ROS), which mediate apoptosis through caspase-3 activation[29]. This phenomenon has been confirmed by improved biocompatibility of GO functionalized with polyethylene glycol (PEG) [30] or dextran [31] when compared to plain GO. However, the molecular mechanisms of graphene oxide-induced cytotoxicity and oxidative stress are still not well elucidated. Previous reports showed that exposures to high concentration of GO can cause a change of cell morphology [28,32]. Depending on GO dose and exposure time, it was demonstrated that GO can produce ROS that can result in the damage of proteins, nucleic acids, and lipids, eventually leading to dysregulation of physiological functions.

Currently, nanomaterials cytotoxicity testing is based on *in-vitro* methods such as MTT assay, for the colorimetric detection of mitochondrial activity, propidium iodide-staining of DNA, as cell death marker, fluorometric detection of ROS generation to evaluate the stress response and colorimetric detection of cytokine secretion for the inflammatory reaction by ELISA method[33]. Several methods have been adopted to analyse the interactions of carbon-based nanomaterials with cells, and transmission electron microscopy represents the gold standard method to evaluate the internalization and localization of GO inside the cells, because it provides high spatial resolution.

Here, we report a study on the interactions of nano-graphene (nGO) at different oxidation degrees low (1); medium (2); and high (3) with live fibroblast cells by varying both its oxidation degree and its concentration in the culture medium. The cell-nGO interaction was investigated by standard assays measuring loss of plasma membrane integrity and, for the first time at best of our knowledge, a peculiar cell nucleus decoration with nano-GO was detected by the digital holography (DH) in microscope configuration[34,35,36]. Mues et al.,[37] have demonstrated the capability of DH as valuable label-free and non-invasive tool for nanomaterial cytotoxicity analysis. DH, compared to standard optical microscopy, provides additional data about cell volume changes, nGO distribution inside/around cells and outer membrane alterations. In particular, DH is a robust technology that is able to furnish a complete characterizations of 3D morphology, as demonstrated for the cases of red blood cells, cancer cells and for the detailed 3D inner structure of auto-

fluorescent chloroplasts of diatoms algae, via quantitative phase imaging without the recurring use of fluorescent imaging[38,39,40]. However, our results show clearly that the cytotoxicity of the nGO decreases systematically by reducing its oxidation degree and its concentration in the cell culture medium and, correspondingly, a peculiar nucleus decoration pattern never reported in literature occurs. Then, based on these considerations, among the nGO with different oxidation degrees, we choose to study by DH the penetration of nGO-2 inside the cells, since it allows obtaining high values of specific surface area, reactive sites for functionalization with drugs, a good adsorption capability of organic molecule as demonstrated by Castaldo et al., and a safe profile on cell viability. Moreover, we monitor the interaction between M-nGO and live cells by measuring the increase of cells' volume after nGO internalization. Definitely, our investigation opens the route for further quantitative characterization of graphene-based nanomaterials and their interactions with more complex biological systems, and for future studies of the use of nGO-2 as a potential nano carrier for drug delivery.

2. EXPERIMENTAL SECTION AND RESULTS

2.1 Quantitative evaluation of nGO uptake in live cells

For a quantitative evaluation of volume changes during nGO uptake in live cells, we used a DH system. The technique allows us to avoid the time-consuming and detrimental procedures usually employed for evaluating the GBM internalization, such as transmission electron microscopy, which requires cell fixing and laborious sample preparation. We focus here our attention on the cell uptake mechanism in case of nGO2 at 50 µg/ml concentration, since it appears to be the best candidate for highly efficient drug delivery for the following reasons. The live/dead tests previously shown demonstrate its biocompatibility, and Castaldo et al.13demonstrated its ability to adsorb an aromatic dye, methylene blue, which can be considered as a model drug.

Figure 1 shows the DH setup based on a Mach-Zehnder interferometer with a CW solide state laser source emitting at 532 nm (max power 400 mW) that is splitted into two beams, the object and the reference. The object beam traverses the sample (i.e. the cells) and, after magnification by a 60☐ water immersion microscope objective (MO) (N.A. 1.2), recombines with the reference beam by the cube beam splitter (BS) and generates an interference fringe pattern (i.e. hologram) such as the one shown in Fig. 1 (c). For a quantitative evaluation of volume changes during nGO uptake in live cells, we used a DH system. The technique allows us to avoid the time-consuming and detrimental procedures usually employed for evaluating the GBM internalization, such as transmission electron microscopy, which requires cell fixing and laborious sample preparation. We focus here our attention on the cell uptake mechanism in case of *nGO2* at 50 µg/ml concentration, since it appears to be the best candidate for highly efficient drug delivery for the following reasons. The live/dead tests previously shown demonstrate its biocompatibility, and Castaldo et al.[13] demonstrated its ability to adsorb an aromatic dye, methylene blue, which can be considered as a model drug.

The cells analysed in the DH system were cultured in a glass WillCo-dish (glass bottom dish, size 35×10 mm, WillCo Wells, The Netherlands), for best optical visualization, and mounted into a conventional micro-incubator chamber (Warner Scientific) in order to maintain the appropriate temperature and atmosphere conditions (37 °C and air mixed at 5% CO_2). A conventional CCD camera (2048×2048 pixels, 5.5 µm pixel, IDS) records the intensity pattern of the hologram that, through numerical processing, gives access to the phase shift information $\Delta\varphi$, arising from the difference in refractive index between the specimen and the surrounding medium [41.]

$$\mathrm{D}j\,(x,y) = \frac{2\rho}{l}\Big[n_c\,(x,y) - n_m\Big]h\,(x,y)$$

where λ is the laser wavelength, $n_c(x,y)$ is the spatial refractive index of the cell, n_m is the refractive index of the surrounding solution, and h is the cell height at position (x,y) in the field of view. It is well known that it is not possible evaluate the cells height without decoupling the contribution of the refractive index of the involved materials, thus we can measure phase volume changes only.

The cells were seeded at a cell density of 5×10^4/mL in a Petri dish (35 mm diameter). After 24 h incubation, a complete DMEM suspension of *nGO2* (ultrasonicated, 50 µg/mL) was added in the Petri and incubated for another cycle of 24 h. Afterwards, the cells were detached by incubation with 0.05% trypsin – EDTA solution (Sigma, St. Louis, MO) for 5 minutes and seeded in the WillCo-dish to be mounted into the DH system. We analysed the cells under both suspended and adherent conditions by recording the holograms just after seeding the cells into the WillCo-dish. Once DH analysis

was completed, the cells were let to adhere on the bottom surface of the Petri dish in the successive three hours. By conventional optical microscopy, we observed that the nanoparticles clearly fill the cytoplasm and maintain the perinuclear localization (Figure 1 d-e), where the cells were not detached after nGO uptake. This demonstrates that the cells, even after trypsinization, preserve the internalized material together with the adhesion functionalities.
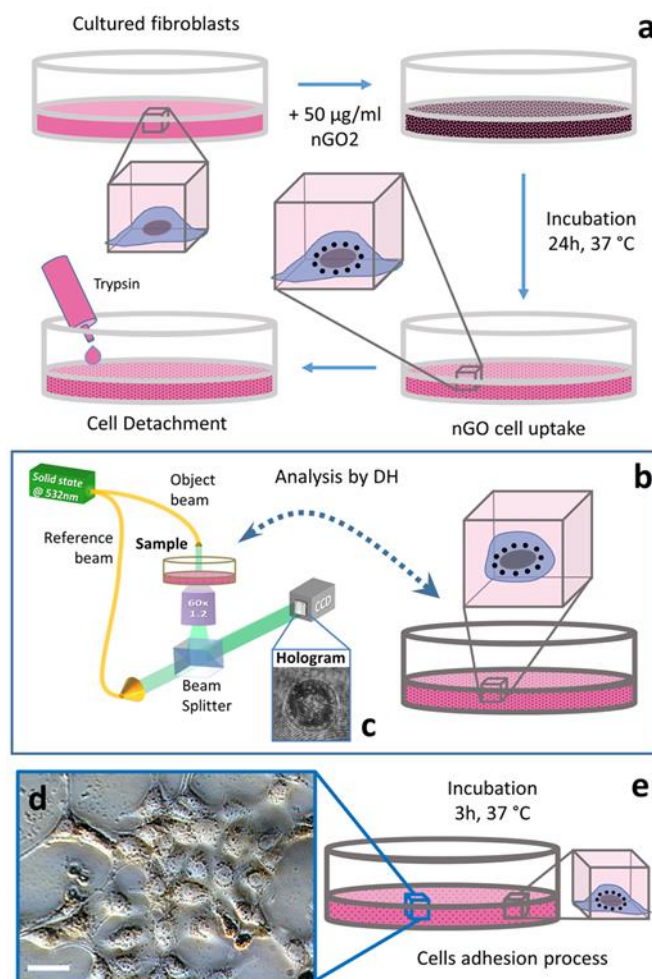

Figure 1. Schematic views of (a) the cell sample preparation for DH analysis and of (b) the DH setup; (c) typical interference pattern recorded by the camera of the DH system. (d) optical microscope image of the cells after 24 h incubation with 50 µg/ml, (e) detached by trypsin and new 3h long incubation in standard medium. [42]

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